The Effect of Mahkota Dewa (*Phaleria Macrocarpa* L.) Leaf Extract Encapsulated in Chitosan Nanoparticles on iNOS and COX-2 Expression in Dextran Sodium Sulphate-Induced Colitis Mice Model

Ari Estuningtyas[1]*, Santi Widiasari[2], Melva Louisa[1], Kusmardi[3]

1. Department of Pharmacology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
2. Student of Biomedical Magister, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
3. Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Abstract

Mahkota Dewa (*Phaleria macrocarpa L*) is indigenous shrub found in Indonesia commonly used in traditional medicine for its anti-inflammatory effects. This study was conducted to assay Mahkota Dewa leaf extract packaged in chitosan nanoparticles provides superior anti-inflammatory effects on ulcerative colitis induced by dextran sodium sulphate (DSS) compared to Mahkota Dewa leaf extract alone.

This study used 36 mice divided into 6 groups: normal, negative control, leaf extract dose 12.5 mg, leaf extract dose 25 mg, leaf extract chitosan nanoparticle dose 6.25 mg, and leaf extract chitosan nanoparticle dose 12.5 mg. Colitis was induced by administering DSS (2 % w/v) through drinking water at 1, 3, and 5 weeks of treatment.

At the end of the study, colon samples were taken from the mice for immunohistochemical analysis; the anti-inflammatory effects of each extract were evaluated by determining the expression of cyclooxygenase-2 (COX-2) and induducible mitric oxide synthase (iNOS) score 0 - 4 in the mouse colon using the image G profiler method.

Mice administered Mahkota Dewa leaf extract at doses of 12.5 and 25 mg and Mahkota Dewa leaf extract in chitosan nanoparticles at doses of 6.25 and 12.5 mg all demonstrated significantly decreased expression of COX-2 and iNOS ($p < 0.001$).

No significant differences between Mahkota Dewa leaf extract and Mahkota Dewa leaf extract in chitosan nanoparticles were observed. Mahkota Dewa leaf extract and Mahkota Dewa leaf extract in chitosan nanoparticles are equally effective at suppressing COX-2 and iNOS expression in mice with DSS-induced colitis.

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Introduction

Inflammation, involved in the pathogenesis of various diseases, is the immune system's response to both infection and injury.[1] It is a complex process that can be initiated by various factors, including bacterial infections and chemically induced irritation caused by injury or cell death. Indeed, tissue injury can lead to the release of inflammatory mediators such as cytokines, tumor necrosis factor-α, and interleukin-1 (IL-1) from leukocytes, monocytes, and macrophages. This process then causes the up-regulation of proinflammatory cytokines, chemokines, and immuneoglobulins, and increases the expression of cellular adhesion molecules. In addition, inflammation also produces a large number of reactive oxygen species (ROS) and increases the expression of phospholipase A2, 5-lipoxygenase (5-LOX), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). ROS themselves also activate nuclear factor kappa B (NFk-B).[2]

Ulcerative colitis (UC), a subtype of inflammatory bowel disease, is characterized by chronic inflammation of the mucous layer of the large intestine. Patients with chronic UC have a high risk of developing colorectal cancer after 10–30 years.[3-6] However, treatments with current drugs, such as corticosteroids and 5-amino-

Corresponding author:
Ari Estuningtyas
Department of Pharmacology,
Faculty of Medicine, Universitas Indonesia,
Jakarta, Indonesia.
E-mail: ari_handoyo@yahoo.com

salicylic acid, may cause serious side effects, including gastric ulcers and gastrointestinal bleeding. Therefore, natural ingredients considered safe for the prevention and treatment of UC have been studied, including Mahkota Dewa (*Phaleria macrocarpa L*) extract.[7,8] The Mahkota Dewa plant has been widely used by people in Indonesia to treat various diseases. These plants contain active substances such as falerin, mangiferin, kaempferol, gallic acid, ikarisida, and other phenolic compounds.[9] In addition, previous studies have shown that Mahkota Dewa leaf extract has a high flavonoid content.[10] Flavonoids are believed to work through several mechanisms, including as antioxidants and direct free radical scavengers, as well as leukocyte immobilization and specific interactions with enzyme systems.[10] We therefore hypothesize it can suppress the inflammatory process involved in ulcerative colitis.

Inflammation in colonic epithelial cells may induce the formation of nitric oxide synthase and COX-2 [11]. COX-2 is an enzyme induced by the COX gene that plays a role in prostaglandin synthesis and has a proinflammatory function.[11] Previous studies have shown that the inhibition of COX-2 decreases inflammation.[12] In addition, iNOS is an enzyme that plays a role in nitric oxide (NO) synthesis. NO is produced in large amounts during chronic inflammation and the expression of iNOS increases in response to cytokine stimulation during inflammatory reactions.[13] In the colon, this process is associated with mucosal lesions, ulceration, intraluminal hemorrhage, dilatation, and overall colonic dysfunction.[14]

Dextran sodium sulfate (DSS) is a polysaccharide sulfate compound widely used to induce inflammation in experimental models related to inflammation of the colon. This model is simple, reproducible, and capable of producing lesions that resemble the human histological features of colitis.[15-17] The flavonoid content of Mahkota Dewa extract has ability to suppress the activity of NF-κB, COX-2, and iNOS on mice colon.[11] Research by Suprapti *et al.*[17] using mice demonstrated that Mahkota Dewa leaf extract at 25 mg suppresses the expression of iNOS, β-catenin, and COX-2 inflammatory proteins. The authors also found administration extract dose killed some of the animals, indicating that it may be toxic to other organs.[17]

The oral administration of Mahkota Dewa leaf extract is believed to suppress the activity of specific inflammatory proteins in the colon. However, this method of administering the extract is also suspected to cause reabsorption, particularly in the stomach and small intestine, before the compound reaches its target at the colon.[10,18-19] Thus, delivering the Mahkota Dewa leaf extract to the target tissue more effectively may result in a more desirable outcome. One method of delivery involves extracting the compound, followed by packing it into chitosan nanoparticles. We hypothesized that the compound would be targeted delivered to the colon where it would then be freed from the chitosan. Ludwig et al[20] study showed that chitosan is one of natural subtances which can be used for mucoadhesive delivery of drugs due to charged polymer chains positively forms electrostatic interactions with negatively charged mucosa. Moreover, the nanosize of the particles may allow the extracted compound to readily diffuse into the colon where it can work to suppress cellular inflammatory proteins. In this regard, a more targeted delivery system may reduce the dose required to elicit anti-inflammatory effects.[21-23]

Materials and methods

This study was conducted at the Animal Laboratory of the Center for Health Research and Development, Ministry of Health, Jakarta, in 2017 with the approval of the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia (17/2/UN2.F1/ETIK/2017).

Animal Study.

The animals used for this study were Swiss Webster male mice, aged around 12 weeks with an average weight of 25 g, obtained from the Animal Laboratory of the Center for Health Research and Development, Ministry of Health, Jakarta. The mice were acclimatized for a week before the start of the experiments. They were housed under standard laboratory conditions at a temperature of 22 ± 2 °C, with a relative humidity of 65 % ± 10 %. A standard pellet rodent diet and water were provided to the animals *ad libitum*. All experimental protocols were approved by the Institutional Animal Ethics Committee of the Universitas Indonesia. Animals

were kept in cages made of materials impermeable to water, strong, easy to clean, and maintained in accordance with the Guidelines for the Treatment and Use of Animal Laboratories of the Animal Committee. The number of the mice was determined by using the Federer formula. The mice divided into 6 groups, 6 mice each, as follows:

1. N group = Normal (not getting treatment).
2. DSS group = Negative control group → the mice only given DSS 2 % w/v with drinking water for 7 days (a week), followed by drinking water without DSS for the next 7 days (a week). These cycles repeated up to 3 times.
3. EMD 25 = Treatment group 1 → the mice also given DSS 2 % w/v with the same cycle to DSS group. At days 8, they also given Mahkota Dewa leaf extract 25 mg/day until the end of the study
4. EMD 12.5 = Treatment group 2 → the mice also given DSS 2 % w/v with the same cycle to DSS group. At days 8, they also given Mahkota Dewa leaf extract 12.5 mg/day until the end of the study
5. NPMD 12.5 = Treatment group 1 → the mice also given DSS 2 % w/v with the same cycle to DSS group. At days 8,
6. NPMD 6.25 = Treatment group 1 → the mice also given DSS 2 % w/v with the same cycle to DSS group. At days 8, they also given Mahkota Dewa leaf extract Chitosan Nano Particle 6.25 mg/day until the end of the study.

To induce colitis, DSS BM 36,000–50,000 was obtained from MP Biomedicals LLC (Solon, OH; Catalog Number: 160110).

The Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract which was purchased from the study center of Biofarmaka IPB (Bogor, Indonesia) and Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract Chitosan Nano Particles was made by PT. Nanotech Indonesia, Tangerang. The extract dose started from 25 mg based on Tati's study and reduced by half. The dosage of extracts in the form of nanoparticles is determined from the dose of ethanol extract which has been reduced by half.

Sample preparation. At the seventh week of the experiments, mice were euthanized by cervical dislocation. The colon tissue of the mice was then excised, cleaned, and rinsed with water. Chunks of the colon tissue were fixed in 10% formalin buffer for 24 – 48 hours and then dehydrated. After the dehydration process, pieces of the colon tissue were inserted into a stratified xylol solution. The next process was the impregnation of colon tissue in paraffin. The tissue was embedded into a paraffin medium on the box cassette that was marked. Paraffin blocks were then cut using a microtome machine (HM 450 sliding microtome) at a thickness of 3 – 5 µm. Pieces were inserted into a water bath (40 – 50 ^{o}C) and then attached to glass slides; glass slides were allowed to dry at 40 ^{o}C for 1 hour.

Immunohistochemical (IHC) staining: COX-2 and iNOS. The 3–5-µm-thick paraffin blocks of colon tissue were attached to a special glass object for the IHC staining technique. The tissue samples were dried at 37 °C and heated to a warmer slide at 60 °C. After the heating process, the samples were deparaffinized and rehydrated. The tissues were then washed in running water for 5 min. After washing, endogenous peroxidase blocking was performed by inserting the tissue into a 0.5 % H_2O_2 solution mixed with methanol for 30 min. The tissue samples were then washed again with running water for 5 min. Next, antigen retrieval was performed. Briefly, the washed tissue was inserted into a container containing a Tris-EDTA solution and then heated in a chamber decloaking device (Decloaking Chamber TM NXGEN) at 96 °C for 10 min. The tissue was then cooled for 45 min, followed by a wash in phosphate-buffered saline (pH 7.4) for 5 min. Sections were then blocked with blocking background sniper for 15 min and incubated for 60 min with primary antibodies against COX-2 [anti-COX2/cyclooxygenase 2 antibody ab23672 (Abcam); diluted to 1 : 1,000] and iNOS [anti-iNOS antibody ab15323 (Abcam); diluted to 1 : 100] in a humidified chamber. After incubation, immunoreactivity was determined with the aid of a horseradish peroxide (HRP)-conjugated secondary antibody (Starr Trek Universal HRP Detection System, Biocare Medical, Catalog Number: STUHRP700 H, L10) for 15 min at 37 °C. The chromogenic reaction was developed with 3,3′-diaminobenzidine solution; a positive signal was defined as a brown color under a light microscope. All images were taken at 400 × magnification. Cells that expressed iNOS or COX-2 negatively contained blue stained nuclei, while the positive cells had a brown – yellow cytoplasm or nuclear membrane.

Interpretation of histochemical staining results. Cells expressing COX-2 and iNOS proteins were calculated from approximately 1,000 colonic epithelial cells from a 5 field-of-view using a cell counter manually in ImageJ software. We used 400 × magnification. Results were analyzed using ImageJ software, which assessed the percentage of the color intensity as either high positive, positive, low positive, or negative; the optical density score was calculated[24,25] using the following formula:

$$\frac{\{(\%\ \text{high positive} \times 4) + (\%\ \text{positive} \times 3) + (\%\ \text{low positive} \times 2) + (\%\ \text{negative} \times 1)\}}{100}$$

From the results of the IHC analysis, data were analyzed using non-parametric statistics. Data were processed using the Kruskal–Wallis test, followed by the Mann–Whitney test. All data were analyzed using the IBM SPSS Statistic Version 20 program.

Results

COX-2 IHC staining. DSS administration significantly increased COX-2 expression in negative groups compared to the normal group ($P < 0.001$). Statistical analysis by the Mann–Whitney test showed significant differences between treatment groups of Mahkota Dewa leaf extract (doses of 25 and 12.5 mg) and Mahkota Dewa leaf extract in Chitosan Nano Particle (doses 12.5 and 6.25 mg) compared with the negative control group ($P < 0.001$). There were significant differences between NMPD 12.5 and N group ($P < 0.001$) and between NMPD 12.5 and NMPD 6.25 group ($P < 0.001$) observed. The COX-2 expression score for each group is shown in Fig. 1. The imunohistochemical staining of COX-2 in mice colon represent on Fig.2.

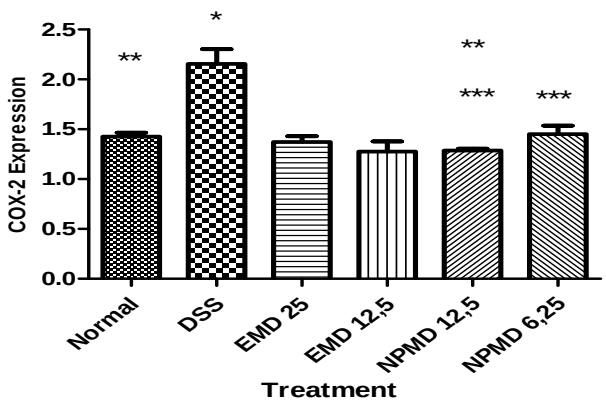

Figure 1. COX-2 expression in mouse colon.
Note: DSS = negative control group; EMD 25 or 12.5 = Mahkota Dewa leaf extract 25 mg or 12.5 mg group; NPMD 12.5 or 6.25 = Mahkota Dewa leaf extract in Chitosan Nano Particle 12.5 mg or 6.25 mg group ; * = significantly difference ($P < 0.001$) between DSS group and other groups; ** = significantly difference ($P < 0.001$) between NPMD 12.5 group and N group; *** = significantly difference ($P < 0.001$) between NMPD 12.5 group and NMPD 6.25 group.

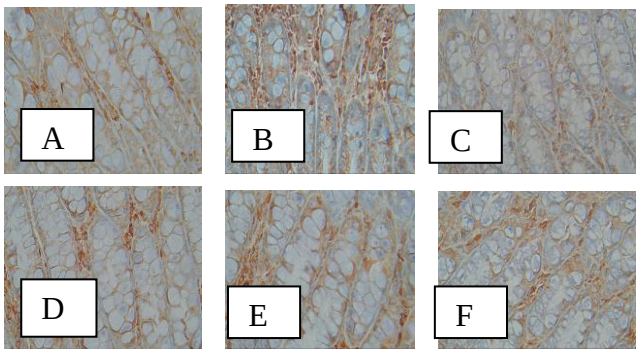

Figure 2. COX-2 immunohistochemical staining in mouse colon.
Note: A = Normal group; B = negative control group; C = Mahkota Dewa leaf extract 25 mg group; D = Mahkota Dewa leaf extract 12.5 mg; E = Mahkota Dewa leaf extract in Chitosan Nano Particle 12.5 mg; D = Mahkota Dewa leaf extract in Chitosan Nano Particle 6.25 mg Black arrow: COX-2 expression in cytoplasm; 400x magnification.

iNOS IHC staining. DSS administration significantly increased iNOS expression in negative control group compared with the normal group ($P < 0.001$). Statistical analysis by the Mann–Whitney test showed significant differences between treatment groups of Mahkota Dewa leaf extract (doses of 12.5 and 25 mg) and Mahkota Dewa leaf extract in chitosan nanoparticles (doses of 6.25 and 12.5 mg) compared with the negative control group ($P = 0.000$). No significant differences between treatment groups of the extract form and nanoparticles of the Mahkota Dewa leaf were

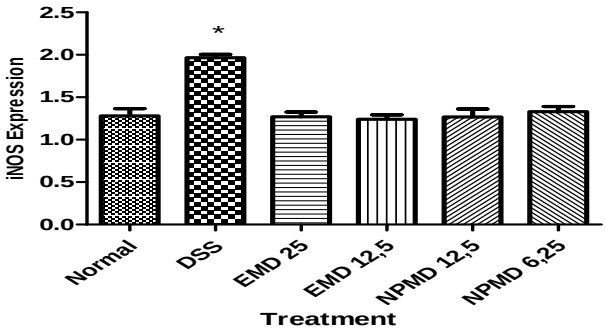

Figure 3. iNOS expression in mouse colon. Note: * = significantly difference (P < 0.001) between DSS group and other groups; observed. The iNOs expression score for each group is shown in Fig. 3. The imunohistochemical staining of iNOS in mice colon represent on Fig.4

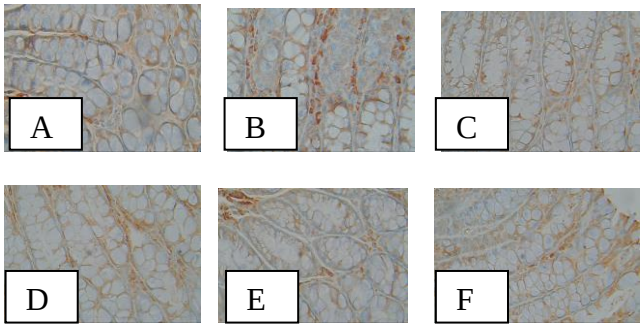

Figure 4. iNOS immunohistochemical staining in mouse colon.
Note: A = Normal; B = DSS; C = EMD25; D = EMD12.5; E = NMPD12.5; D = NMPD6.25 mg Black arrow: iNOS expression in cytoplasm; 400x magnification.

Discussion

Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract as reported by Hendra R et al[8] and Lee et al.[26] is known contain several flavonoids such as kaemferol, routine, naringin and miresetin. This flavonoids have several effects like antioxidant, radical scavenger, leukocyte immobilization, and interaction with enzyme.[26]

Dextran sodium sulfphate is a dextran polyamine which has been shown affect to the barrier function of the colonic mucosa. The disrupt its permeability will cause cell damage and subsequently trigger an immune response. Administration of DSS 2% for 7 days in 3 repetitive cycles refers to Zhang et al[27] study to make chronic colitis in animal model. Various studies, have shown that the use DSS for colon cancer induction in animal models can increase COX-2 expression.[28,29] DSS will cause damage to the epithelial mucosal barrier function, which allows entry of bacteria and antigens into the colonic mucosa, and causes a severe inflammatory response.[29] Inflammatory cells can induce the COX-2 enzyme that plays a role in converting arachidonic acid to prostaglandin E2 (PGE2) which is the most important in the inflammatory response.

In this study, Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract was made in the form of nano-chitosan particles with aim to the more targeted delivering extracts in colon, thereby increasing the effectiveness of the drug. The reason that the extract is more targeted to the workplace in the colon is based on Tati et al[17] research where the extract has been shown to have anti-inflammatory effects on dose 25 mg and 50 mg. This effect increases with increasing the dose. But on that study the survival rate of animals for both doses were decreased compare to the normal group and control positive group. Increasing the dose shown increase in animal mortality too. Researchers suspect that Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract given orally also affects other organs and is likely to cause side effects or toxic effects that cause death in experimental animals.

One thought of using nanoparticles is it can help increase drug concentration in desired tissues while minimizing drug concentrations in other unwanted tissues through passive targeting. Nanoparticles made with polymers can be used for targeted delivery systems, increase bioavailability and controlled release. Nanoparticle polymers commonly used as a conductor to make natural materials are chitosan and Na TPP, the positive charge of chitosan amine groups will interact with negative charges TPP forms complexes with sizes in the range of nanoparticles.[28] Chitosan can be used for mucoadhesive delivery of drugs due to charged polymer chains positively forms electro-static interactions with negatively charged mucosa.[20] In addition, it can increase drug absorption through the paracellular route by opening epithelial cell tight junctions[25] Nanoparticles for medical purposes usually measure < 200 nm (ie microcapillary width).[23]

Determination of the dose of Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract is based on the smallest effective dose that has an antiinflammatory effect on the research of Tati et

al[17], which is 25 mg and then reduced by half that is 12.5 mg. The dose of Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract in chitosan nanoparticles starts from dose 12.5 mg and reduced by half of 6.25 mg. The dose starts 12.5 mg because researchers suspect the reduced particle size will have the same effect as the Mahkota Dewa leaf extract with dose 25 mg in normal size. In addition, based on the results of the volume ratio analysis, the volume of Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract with Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract in nanoparticle chitosan was 1: 7, so to compare the appropriate dose volume of extract in the form of chitosan nanoparticles was 7 times compared to ordinary extract. The administration of large doses will cause a large volume of the test substance given to exceed the gastric capacity of the experimental animal. Therefore the dose of extract in chitosan nanoparticles starts from 12.5 mg.

Observation of COX-2 and iNOS expression in colon proved that Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract and Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract in chitosan nanoparticles were able to inhibit inflammation in mouse colon induced DSS, compared with negative control group. However, the results of the comparison among the treatment groups were not significant, this is probably due to the size of the nano particles made still exceeding 200 nm. Results of measurements of Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract in Chitosan Nano Particles ranges from 500 - 700 nm.

Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract can reduce COX-2 protein expression, this is probably due to COX-2 suppression activity by flavonoids of Mahkota Dewa (*Phaleria macrocarpa L*) leaf extract containing kaemferol, routine, naringin and miresetin. As reported in the Lee et al[26] study, kaemferol can suppress UVB-induced COX-2 expression through activity inhibition of Src kinase, a non-receptor protein tyrosine kinase marker of cancer progression that works phosphorylation of certain tyrosine residues in other proteins. Routine suppression of COX-2 expression was also reported in a study by Choi et al[30]. Wen-Jing et al[31] reported that naringin was able to decrease COX-2 expression in the human line cells of human cervical cancer and Lee et al[32] reported that mirisetin could reduce COX-2 expression through blockade of activity. NF-κB binding is assessed using an electrophoresis mobility test. COX-2 expression in normal groups was characterized by a score of 1.427. This is consistent with studies conducted by Jackson et al[33] in normal, inflamed and gastric gastric mucosae, where it is known that COX-2 is expressed on normal tissue, and then there is an increase in expression in inflammatory tissue and in ulcer tissue.

The highest iNOS expression is in the induced group. This is because the administration of DSS can induce iNOS expression. In inflammatory reactions, the induced form of NO synthase (iNOS) has a considerable role. Inflammatory cells express iNOS in response to cytokine stimulation such as IL-1β, IL-6 in inflammatory reactions. These enzymes choke NO production which has a proinflammatory action namely vasodilator, increasing permeability. Suzuki's study et al[34] showed that immunehis-tochemistry, nitrothyrosine observations were greater in 2% and 1% DSS-induced mice. Nitrothyrosine is a marker of the reaction between peroxinitrite and protein tyrosine. Peroxinitrite is the result of the reaction of NO with superoxide (O2).[28] The high peroxy nitrite shows the high nitric oxide (NO) which proves the presence of iNOS as an enzyme that plays a role in the production of NO.[29] In this study normal animals also expressed iNOS protein with a score of 1[29], this is consistent with the study of Robert et al[35] which proved that iNOS was expressed on the surface of human colonic epithelium that did not experience inflammation. Inducible Nitric oxides may be induced by local luminal factors such as lipopolysaccharide (endotoxins) bacteria, the production of nitric oxide is an oxidative barrier that continuously reduces bacterial translocation so that it serves as a defense against pathogenic microorganisms.

The results of this study prove that Mahkota Dewa leaf extract can suppress the expression of COX-2 and iNOS in DSS-induced mice colon. Interestingly, from the results of this study, extracts in smaller doses of nano particles can provide an suppressive effect of COX-2 and iNOS expressions comparable to ordinary extracts. This proves that the extract packaged in Chitosan Nano Particles makes the delivery of the test substance more targeted and reaches the targeted tissues better.

Conclusions

Mahkota Dewa leaf extract and Mahkota Dewa leaf extract in Chitosan Nano particle both can suppress inflammation in mice epithelial colon induced by DSS, indicated by decreased expression of inflammatory proteins COX-2 and iNOS.

All doses of the Mahkota Dewa leaf extract and Mahkota Dewa leaf extract in Chitosan Nano particle gave a significant results in decreased the expression of COX-2 and iNOS in mouse colon epithelial crypts induced by DSS ($P < 0.001$).

Mahkota Dewa leaf extract in Chitosan Nano particle provides promising result because at smaller dose (6.25 mg) gives as good results as extract alone (25 mg and 12.5 mg).

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Conflict of interests

The authors declare no conflicts of interest.

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